Association of *UCP1*, *UCP2* and *UCP3* gene polymorphisms with cardiovascular disease risk factors in European adolescents: the HELENA study

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ABSTRACT

Introduction: Cardiovascular diseases (CVDs) are responsible of 31% of all deaths worldwide. Genetic predisposition to CVDs in adolescents remains largely unknown.

Objective: To examine the association of *UCP1*, *UCP2* and *UCP3* gene polymorphisms with CVDs risk factors in European adolescents.

Study design: A cross-sectional study that involves 1.057 European adolescents (12-18 years old) from the HELENA Study. A total of 18 polymorphisms of *UCP1*, *UCP2* and *UCP3* genes were genotyped. We measured serum total cholesterol, HDL, ApoA1, ApoB, leptin, triglycerides, glucose, insulin, blood pressure, and calculated HOMA (homeostatic model assessment) and a CVD Risk Score.

Results: The G allele of *UCP2* rs2735572 and T allele of *UCP2* rs17132534 were associated with higher diastolic blood pressure (P=0.001; FDR=0.009 and P=8e−04; FDR=0.009, respectively). We observed that the AATAG haplotype of *UCP1* was associated with higher serum ApoB/ApoA1 (P= 0.008; FDR = 0.031) and ApoB levels (P= 0.008; FDR = 0.031). Moreover, the ACC haplotype of *UCP3* was associated with a higher CVD risk score (P= 0.0036; FDR = 0.01).

Conclusions: Two *UCP2* polymorphisms and haplotypes of *UCP1* and *UCP3* were associated with CVD risk factors. These findings suggest that *UCPs* may have a role in the development of CVD already in adolescents.

Introduction

Cardiovascular diseases (CVDs) are the main cause of premature death and chronic disability worldwide (1). CVD events occur most frequently during or after the fifth decade of life, however, there is evidence indicating that the precursors of CVD have its origin in the first decades of life (2). Therefore, prevention is fundamental to reduce the incidence of these pathologies, especially in young people.

CVDs are a result of complex harmful interactions between environmental and genetic risk factors. Environmental factors include unhealthy diet, tobacco use or physical inactivity (3). However, efforts have been insufficient to decrease the prevalence and new pathogenic dimensions come into play. Recent studies have described the association between some single nucleotide polymorphisms (SNPs) with myocardial infarction (4) and other cardiovascular complications (5). For example, uncoupling protein genes (UCPs) have been associated with risk factors of cardiovascular disease such as prediabetes and type 2 diabetes mellitus (T2DM) (6), overweight and obesity (7,8), plasma levels of cholesterol (9) or hypertension (HT) (10) , mainly in adults over 50 years of age.

The most studied UCP genes are: i) *UCP1*, which main function is heat production through non-shivering thermogenesis in brown adipose tissue (BAT) (11); ii) *UCP2*, in addition to may have a regulating role in thermogenesis of BAT, seems to be involved in the control of reactive oxygen species (ROS) production (12,13), the modulation of insulin secretion (14) and the regulation of mitochondrial fatty acid oxidation (15), and iii) *UCP3,* which role has been related to the coupling regulation of mitochondrial respiration in skeletal muscle mitochondria (16) and fatty acids oxidation (17), and is a mediator of thermogenesis (18). The playing role of uncoupling proteins in human physiology makes UCPs ideal targets against

cardiovascular-associated pathologies. Indeed, several SNPs of *UCP2* (rs660339, rs659366) (19,20) and *UCP3* (rs2075577, rs3781907, rs1800006, rs1800849) (19,21–23) have been associated with T2DM, overweight/obesity, serum total and LDL-cholesterol and others cardiovascular risk markers. Nevertheless, poor evidence of CVD risk factors, especially in youth has been described.

Data obtained within the Healthy Lifestyle in Europe by Nutrition in Adolescence Cross-Sectional Study (HELENA-CSS) study provide an excellent opportunity to study the association of *UCP1*, *UCP2* and *UCP3* SNPs with CVD risk factors in European adolescents. The HELENA study was designed to provide reliable data on nutrition and health-related variables in a relatively large sample of European adolescents from 9 different countries and includes information on 18 SNPs of *UCP1*, *UCP2* and *UCP3* genes as well as a number of CVD risk factors. To our knowledge, UCPs polymorphisms have not been identified in GWAS of body weight or body composition in adults or other age groups

The aim of this study was therefore to examine the association of 18 *UCP1*, *UCP2* and *UCP3* SNPs (see Table 1) with CVD risk factors in European adolescents.

Material and methods

Participants

The HELENA-CSS attempted to report the lifestyle and nutritional status of European adolescents. A total of 3865 participants (12-18 year old) of nine European countries (Greece, Germany, Belgium, France, Hungary, Italy, Sweden, Austria and Spain) were selected to be part of this study. They were randomly selected from public and private schools in each city between October 2006 and December 2007. We collected blood samples of one-third of these participants (N=1155) with the consequent genetic analysis and clinical biochemistry assays. Finally, 1057 (552 girls) adolescents with UCPs SNPs and CVD risk factors data were included in this study. Adolescents and corresponding parents/guardians were fully informed about aims and methods of the study such as inclusion criteria (24,25), and signed an informed written consent. Ethical guidelines of the Declaration of Helsinki 1964 (revision of Edinburgh 2000), Good Clinical Practice, and legislation about clinical research in humans in each of the participating countries were respected by the study. Human research committees of each center involved approved the protocol (26).

Assessment of Cardiovascular Risk Factors

A total of 30 ml of blood samples were drawn after a 10-h overnight fast at school between 0830 and 0900 hours following a standardized blood collection protocol. Serum/plasma was centrifuged directly at the schools at 3500 rpm (for 15 min, at room temperature). After centrifugation they were stored and transported (4-7ºC) to the central laboratory (Bonn, Germany) where they were deposited at -80ºC as explained in detailed elsewhere (27). Serum concentrations of cardiovascular risk factors were measured in centralized laboratories.

The CVD risk factors analysed included serum total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL), ApoA1, ApoB, leptin, triglycerides and glucose, which were measured on the Dimension RxL clinical chemistry system (Dade Behring, Schwalbach, Germany) with enzymatic methods. Insulin was measured by a solid-phase two-site chemiluminescent immunometric assay with an Immulite 2000 analyzer (DPC Biermann GmbH, Bad Nauheim, Germany). Homeostasis model assessment (HOMA) was calculated ([glycaemia X insulin]/22.5) as resistance to insulin indicator, along with the Quantitative Insulin Sensitivity Check Index (QUICKI), which was calculated as 1/[log(insulin) + log(glycaemia)].Blood pressure was measured with an automatic oscillometric device (OMRON M6). Adolescents quietly sat for 5 min before the measurements, conducted on the right arm in an extended position. Two measures of diastolic and systolic blood pressure (DBP and SBP, respectively) were taken 5 min apart, and the mean of both values (in mmHg) was used in analyses.

We computed a CVD risk score with the mean of the standardize value [(value- mean)/standard deviation] of the following variables: Total cholesterol/HDL, triglycerides, HOMA, systolic blood pressure, and triceps and subscapular skinfolds (28). The characteristics of the study sample, including CVD risk factors, are shown in Table 2.

Genotyping

Blood for DNA extraction was collected in EDTA K3 tubes, stored at the Analytical Laboratory at the University of Bonn, and then sent to the Genomic Analysis Laboratory at the Institut Pasteur de Lille (Lille, France). DNA was extracted from

white blood cells with the Puregene kit (QIAGEN, Courtaboeuf, France) and stored at 20°C. Samples were genotyped by an Illumina System (Illumina, Inc, San Diego, California) and the software used was GoldenGate (Inc, San Francisco, California). High rate of genotyping success was performed (≥97,8%) and each polymorphism respected the Hardy-Weinberg equilibrium (P>0.2 in all cases; Table 1). Several polymorphisms of the same genes showed linkage disequilibrium between them (Figures 1 and 2).

Statistical analysis

Deviations from Hardy-Weinberg equilibrium (HWE) were determined by means of an exact test and considering a p value of 0.05 as a threshold. Associations between genetic markers and CVD risk factors were assessed through linear models. Five inheritance models (dominant, recessive, log-additive, codominant and overdominant) were used for all analyses, except in those where rs2071416, rs2735572 and rs17132534 SNPs were involved. These polymorphisms were analysed using only a dominant model due to the low number of minor homozygotes (minor allele frequency < 0.1; Table 1). Previous findings shown the association between non-additive models with UCPs, which indicates the interest of perform this five models and compare the additive models with non-additives ones (29). Adjustment variables were body mass index (calculated as weight in kilograms divided by height in meters squared), age, gender and center. For each SNP, P values were computed using the likelihood ratio test (LRT) between a model with the polymorphism and a null model without it. These analyses were performed with the "SNPassoc" R package (30). We considered the associations between all SNPs and each phenotype under a given heritage model such as the family test, i.e. the number of tests were equal to the

number of SNPs analysed for a given phenotype. We selected the significant genotype-phenotype associations to perform haplotype analysis, i.e. only SNPs and phenotypes significant associated were considered for next analyses. Given the exploratory nature of these analyses and the reduced number of independent tests (markers are in linkage disequilibrium), the Bonferroni correction could be too conservative (31). Instead of this method, we performed an exploratory selection of associations using an approach that controls the expected proportion of false positives (False Discovery Rate [FDR]) (32). Therefore, associations with FDR < 0.1 were used in haplotype analyses.

Linkage disequilibrium between polymorphisms and haplotype block structures were evaluated with Haploview 4.2 (http://www.broad.mit.edu/mpg/haploview) and the R package haplo.stats (33). First, haplotype blocks were generated by the algorithm of four-gamete rules (34) using Haploview. For each block, we tested if the observed frequencies of haplotypes were deviated from those expected under linkage equilibrium using "haplo.em" {haplo.stats} (P = 0 in all blocks). Finally, we assessed the association between haplotypes and phenotypes by means of a permutation procedure performed with haplo.score {haplo.stats}. Only additive and dominant models were considered given the low frequency of some haplotypes. For those significant associations we performed regressions between haplotypes and phenotypes with the purpose of testing significant differences between haplotype levels. These regressions were performed with haplo.glm {haplo.stats}. Again, the FDR was calculated from the P values for differences between the reference haplotype (the most frequent) and other haplotypes.

Results

Association between UCP polymorphisms and CVD Risk Factors

Two *UCP2* SNPs were individually associated with CVD risk factors after multiple-comparison corrections with a FDR<0.05 threshold. Associations with FDR<0.1 were also selected as a screening method to further haplotype analyses (Figure 3). All individual comparisons between genotypes and CVD risk factors are presented in supplementary file. We observed that the G minor allele of rs2735572 SNP and the T minor allele of rs17132534 SNP were associated with higher DBP under a dominant inheritance model (beta coefficient=0.04, P=0.001; FDR=0.009 and beta coefficient=0.04, P=8e−04; FDR=0.009 respectively; beta coefficients obtained from models with the response variable log transformed; Figure 3).

Association between UCP polymorphism haplotypes and CVD risk factors

UCP1 block contains the rs12502572, rs11932232, rs6822807, rs6818140, and rs2071416 SNPs (Figure 1). The AATAG haplotype of *UCP1* was significantly associated with a higher ApoB/ApoA1 ratio than the reference GATAT haplotype (global P=0.046; difference between groups =0.06; 95CI = 0.02 - 0.10; P= 0.008; FDR = 0.031; under additive model; differences between groups obtained from models with the response variable log transformed, respectively). The AATAG haplotype was also associated with higher ApoB levels than the reference GATAT haplotype (global P=0.045; difference between groups =0.06; 95CI = 0.02 - 0.10; P= 0.008; FDR = 0.031; under additive model). Also an association between the *UCP3* block 2 (rs7930460, rs2075577 and rs2734828, Figure 2) and the risk score was observed. The ACC haplotype was associated with a higher risk score compared with

the ATC haplotype (global P=0.008; difference between groups= 0.06; 95CI = 0.02 - 0.10; P= 0.0036; FDR = 0.01; under dominant model).

Discussion

We observed that the *UCP2* G and T alleles of the rs2735572 and rs17132534 SNPs were associated with higher diastolic blood pressure in European adolescents. Moreover, we found that the *UCP1* AATAG haplotype was associated with higher serum ApoB/ApoA1 and ApoB levels. Finally, the *UCP3* ACC haplotype was associated with a higher CVD risk score. Taken together, these findings suggest that UCPs may have an important role in cardiovascular health already in the first decades of life.

To our knowledge, this is the first study investigating the association between UCPs and CVD risk factors in European adolescents. Several studies with smaller sample sizes (6,21,35,36) reported an association of single polymorphisms of *UCP3* SNPs or combined haplotypes of *UCP2/UCP3* with several CVD risk factors such as TC and LDL levels, insulin or HOMA in adults. In contrast, we observed no association of these UCPs SNPs with CVD risk factors in European adolescents. These discordances may be due to the fact that they are population dependent, with possibly different allele frequencies and penetrance in these populations. Also differences in age, inter-country differences in lifestyle behaviours and sample sizes are important and could lead to differences across studies.

A plausible mechanism to partially explain the observed associations is that polymorphisms or haplotypes of *UCP*s could alter UCP functions and predispose to cardiovascular risk or an increased Risk Score (due to it is make up of cardiovascular risk factors). This dysfunction may explain the phenotypes observed with CVD risk

through, i) dysfunction of the process of oxidation of fatty acids, leading to altered serum lipid levels as TC, LDL, HDL or TG (9,15), ii) in relation to ROS regulation mediated by UCP2; Pierelli *et al*. (37) showed that knockout mice deletion of the *UCP2* gene contributes to atherosclerosis lesion development and a significantly shorter lifespan. Several studies in humans and cultured cells suggested that excessive ROS production is involved in the atherosclerotic plaque formation and progression (38,39). Therefore, evidence suggests that decreasing ROS production is a remarkable target to prevent the atherosclerotic process. *UCP2* negatively regulates intracellular ROS production (12,13) making it a potential therapeutic target for the treatment of vascular diseases, iii) UCPs have been also related to blood pressure control. Dhamrait et al. (40) described the role of UCPs in the regulation of angiotensin-converting enzyme (ACE). This enzyme plays a pivotal component of the endocrine renin–angiotensin system (RAS), also playing a key role in the regulation of the human circulation. ACE favours the rise of angiotensin II and aldosterone, leading to salt and water retention by the kidney and to constriction of small blood vessels in the arterial tree. Taken together, these actions serve to elevate blood pressure. Moreover, Dhamrait et al. (40) showed that some *UCP3* and *UCP2* SNPs were associated with higher age-adjusted ACE activity, which could contribute to a hypertension status, which is also consistent with our findings. Cardiovascular diseases are pathologie s with a long-term latency period and modifiable cardiovascular risks factors, which makes prevention fundamental, especially in these populations with genetic predisposition.

A limitation of our study is its cross-sectional nature. Our results should be considered carefully and studies with larger sample size could help to further confirm this possible genetic predisposition.

In conclusion, we observed an association between the *UCP2* rs2735572 and rs17132534 SNPs with higher diastolic blood pressure in adolescents from nine European countries. We also observed a haplotype association of *UCP1* and *UCP3* with higher blood apolipoproteins levels and risk score, respectively. These findings suggest that *UCPs* may have an important role in the development of CVD predisposition already in European adolescents.

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Figure Legend

- **Figure 1**. Haploview result belonging to blo ck 1 of *UCP1* polymorphisms, which contains (rs12502572, rs11932232, rs6822807, rs6818140 y rs2071416), according to genotyping data of this study. Boxes number referred to linkage disequilibrium (D') between SNPs, boxes with no number means 100% linkage (D' = 1). Colour legend: i) Bright red = high D'; White = low D' (see Haploview documentation for further details; http://www.broad.mit.edu/mpg/haploview).
- **Figure 2**. Blocks 1, 2 and 3 of *UCP2* and *UCP3* polymorphisms, which contains rs2735572, rs660339, rs17132534 and rs659366 SNPs for block 1 (*UCP2*); rs7930460, rs2075577 and rs2734828 SNPs for block 2 (*UCP3*); rs1800006 and rs1800849 SNPs for block 3 (*UCP3*), according to genotyping data of this study. Boxes number referred to linkage disequilibrium (D') between SNPs, boxes with no number means 100% linkage (D' = 1). Colour legend: i) Bright red = high D'; White = low D' (see Haploview documentation for further details; http://www.broad.mit.edu/mpg/haploview).
- **Figure 3**. Significant associations between phenotypes and UPC SNPs (FDR<0.1). For each association, the phenotypes and markers implicated are shown, along with the inheritance model, P value and FDR. Values are adjusted for body mass index, center, sex, and age.
- **Figure 3 (continuation)**. Significant associations between phenotypes and SNPs (FDR<0.1). For each association, the phenotypes and markers implicated are shown, along with the inheritance model, P value and FDR. Values are adjusted for body mass index, center, sex, and age.

Table 1: Frequency of the major and minor alleles is shown, along with the corresponding frequency of genotypes. In addition, sample size and results of exact test to assess deviations from Hardy-Weinberg equilibrium (HWE) are presented.

	Major allele	Minor allele	Major allele freq.	Minor allele freq.	Major homozygous freq.	Heterozygous freq.	Minor homozygous freq.	Sample size	pHWE
UCP1									
rs2071415	T	C	0.85	0.15	0.73	0.25	0.03	1057	0.39
rs7688743	G	A	0.81	0.19	0.65	0.32	0.04	1057	0.92
rs6818140	A	G	0.81	0.19	0.65	0.32	0.03	1057	0.32
rs6822807	T	C	0.73	0.27	0.53	0.4	0.07	1056	0.59
rs11932232	A	G	0.81	0.19	0.65	0.32	0.03	1057	0.32
rs12502572	G	A	0.64	0.36	0.42	0.45	0.13	1057	0.74
rs6536991	T	C	0.73	0.27	0.53	0.4	0.07	1057	1
rs2071416	T	G	0.92	0.08	0.84	0.15	0.01	1057	0.31
UCP2									
rs2735572	G	A	0.94	0.06	0.88	0.12	0	1057	0.58
rs17132534	T	C	0.94	0.06	0.88	0.12	0	1057	0.79
rs660339	C	T	0.61	0.39	0.38	0.47	0.15	1034	0.65
rs659366	C	T	0.64	0.36	0.42	0.46	0.13	1057	0.84
UCP3									
rs7930460	A	G	0.79	0.21	0.62	0.33	0.05	1057	0.78
rs2075577	T	C	0.53	0.47	0.27	0.52	0.21	1057	0.22
rs2734828	C	T	0.75	0.25	0.56	0.37	0.06	1057	0.81
rs3781907	T	C	0.74	0.26	0.54	0.39	0.07	1057	0.75

Table 2: Mean ± SD of cardiovascular risk factors.

Phenotype	All (n=1057)	Male (n=505)	Female (n=552)
Age (years)	14.71 ± 1.22	14.74 ± 1.25	14.68 ± 1.2
Weight (kg)	58.72 ± 12.67	61.86 ± 14.29	55.85 ± 10.17
Height (cm)	165.46 ± 9.34	169.5 ± 9.91	161.76 ± 6.98
BMI (kg / m^2)	21.34 ± 3.67	21.39 ± 3.99	21.3 ± 3.37
Cholesterol (mg/dL)	160.74 ± 27.69	154.03 ± 26.13	166.88 ± 27.68

HDL (mg/dL)	55.26 ± 10.67	53.17 ± 10.12	57.17 ± 10.81
LDL (mg/dL)	94.49 ± 25.09	90.78 ± 24.32	97.89 ± 25.33
Triglycerides (mg/dL)	69 ± 35.09	64.13 ± 31.65	73.46 ± 37.45
LDL/HDL	1.78 ± 0.63	1.78 ± 0.65	1.78 ± 0.6
Cholesterol/HDL	2.99 ± 0.66	2.98 ± 0.69	2.99 ± 0.63
Triglycerides/HDL	1.33 ± 0.88	1.29 ± 0.83	1.37 ± 0.92
ApoA1(mg/dL)	1.5 ± 0.22	1.46 ± 0.21	1.55 ± 0.23
ApoB (mg/dL)	0.65 ± 0.16	0.63 ± 0.15	0.68 ± 0.16
ApoB/ApoA1	0.44 ± 0.13	0.44 ± 0.13	0.45 ± 0.13
apoB/LDL	0.27 ± 0.03	0.27 ± 0.03	0.27 ± 0.03
Leptin (ng/ml)	19.61 ± 22.19	9.55 ± 14.21	28.36 ± 24.11
Insulin (micro IU/mL)	10.31 ± 7.79	10.16 ± 8.82	10.46 ± 6.7
HOMA	2.35 ± 1.96	2.36 ± 2.24	2.34 ± 1.65
QUICKI	0.35 ± 0.03	0.35 ± 0.03	0.35 ± 0.03
SBP (mm Hg)	120.03 ± 13.3	124.16 ± 13.93	116.29 ± 11.5
DBP (mm Hg)	68.03 ± 8.84	67.52 ± 8.91	68.49 ± 8.76
CVD Risk score	-0.01 ± 0.61	-0.03 ± 0.66	0.02 ± 0.56

BMI: body mass index (calculated as weight in kilograms divided by height in meters squared) HDL: high density lipoprotein; LDL: low density lipoprotein; Apo: apolipoprotein; HOMA: homeostatic model assessment; QUICKI: quantitative insulin sensitivity check index; SBP: systolic blood pressure; DBP: diastolic blood pressure

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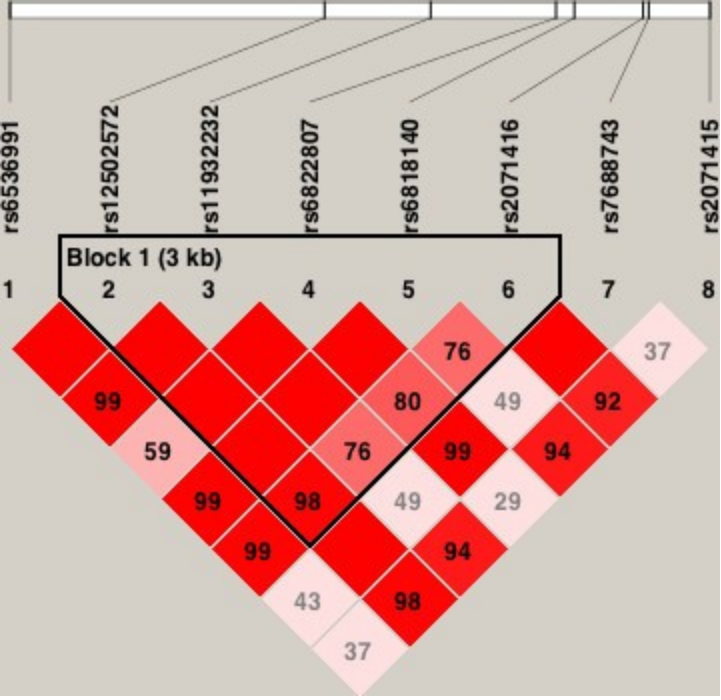
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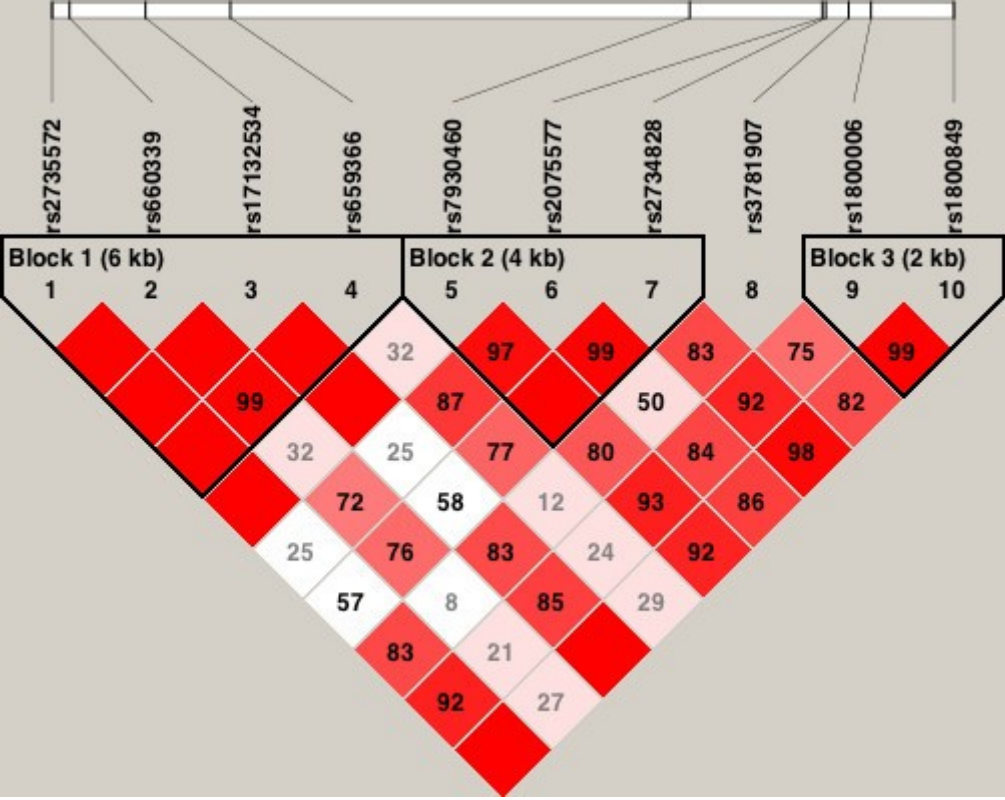
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1
2
3
4
Block 2 (4 kb)
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6
7
8
Block 3 (2 kb)
9
10
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32
97
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99
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25
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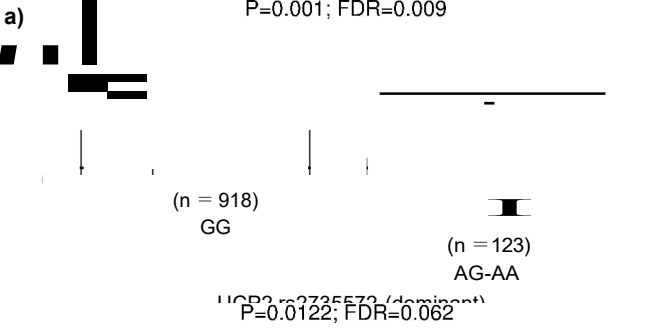
83
21
92
27

a)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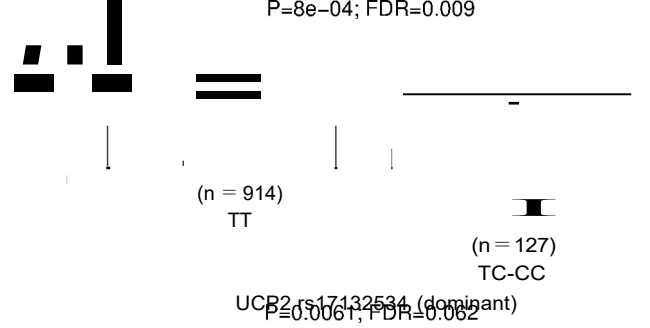
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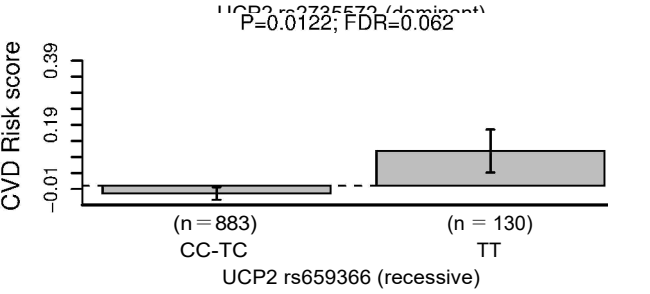
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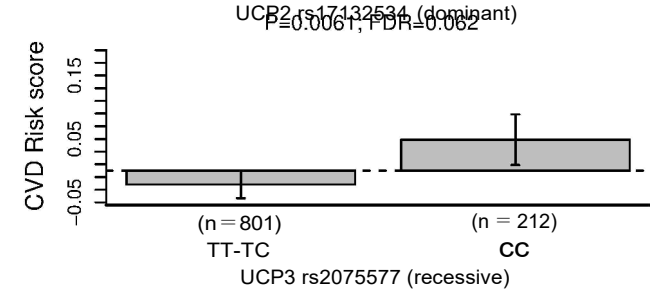
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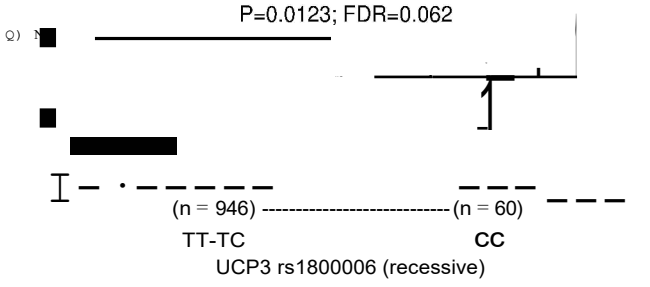
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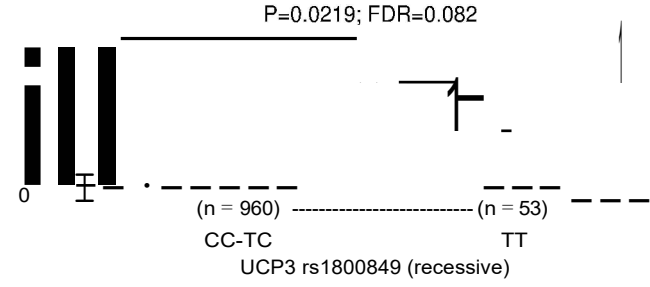
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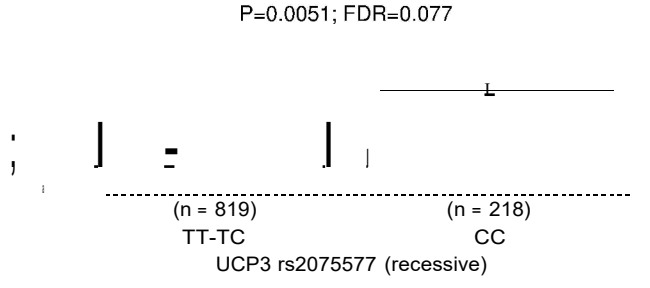
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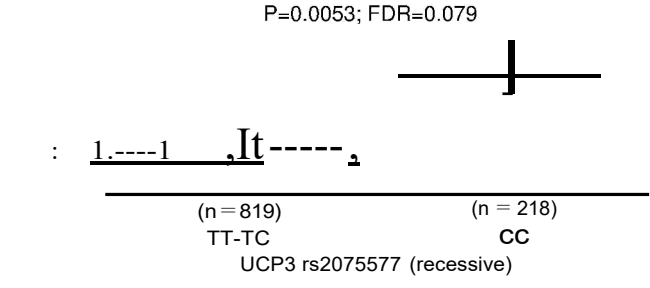
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TT-TC
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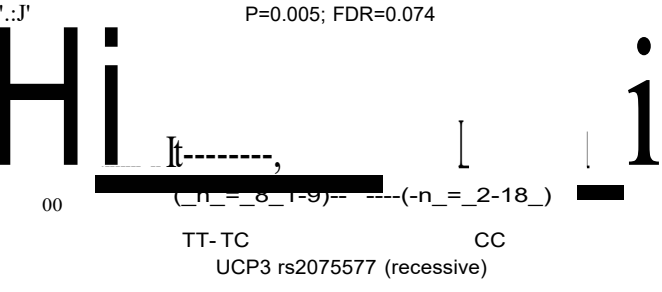
CC
UCP3 rs2075577 (recessive)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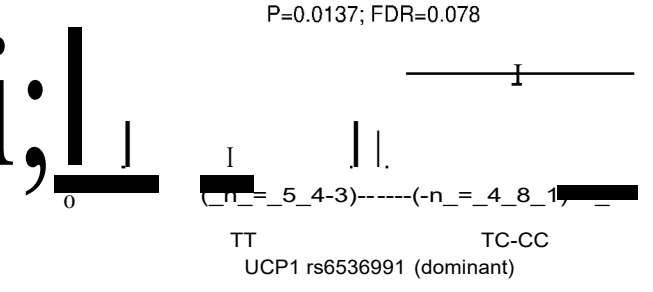
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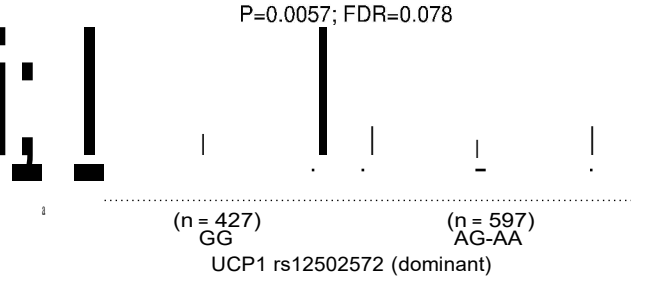
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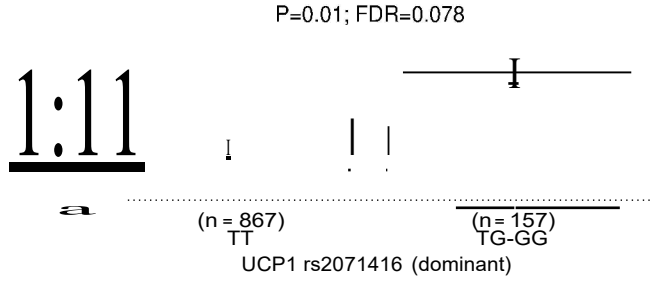
AG-AA
UCP1 rs12502572 (dominant)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TG-GG
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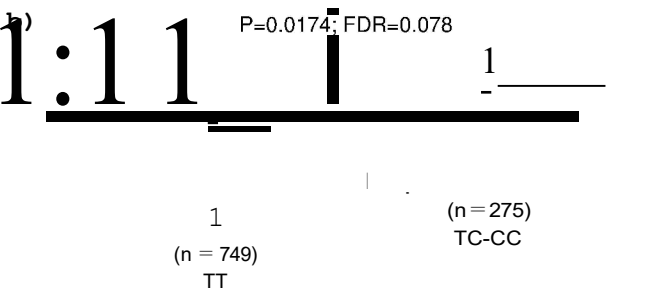
b)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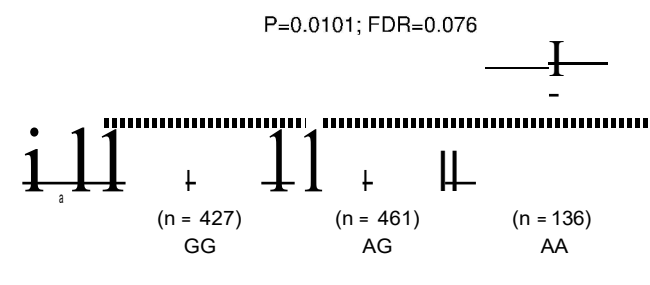
UCP1 rs2071415 (dominant)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AG
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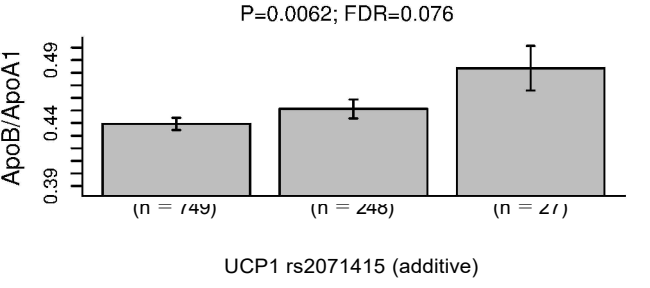
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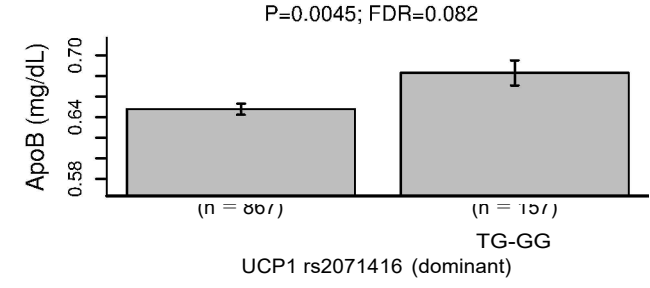
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ApoB (mg/dL)
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0.58
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(n = 157)
TG-GG
UCP1 rs2071416 (dominant)